

Study of regulation of mitochondrial respiration in vivo An analysis of influence of ADP diffusion and possible role of cytoskeleton

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Abstract

The purpose of this work was to investigate the mechanism of regulation of mitochondrial respiration in vivo in different muscles of normal rat and mice, and in transgenic mice deficient in desmin. Skinned fiber technique was used to study the mitochondrial respiration in the cells in vivo in the heart, soleus and white gastrocnemius skeletal muscles of these animals. Also, cardiomyocytes were isolated from the normal rat heart, permeabilized by saponin and the “ghost” (phantom) cardiomyocytes were produced by extraction of myosin with 800 mM KCl. Use of confocal immunofluorescent microscopy and anti-desmin antibodies showed good preservation of mitochondria and cytoskeletal system in these phantom cells. Kinetics of respiration regulation by ADP was also studied in these cells in detail before and after binding of anti-desmin antibodies with intermediate filaments. In skinned cardiac or soleus skeletal muscle fibers but not in fibers from fast twitch skeletal muscle the kinetics of mitochondrial respiration regulation by ADP was characterized by very high apparent K_m (low affinity) equal to 300–400 μ M, exceeding that for isolated mitochondria by factor of 25. In skinned fibers from m. soleus, partial inhibition of respiration by NaN_3 did not decrease the apparent K_m for ADP significantly, this excluding the possible explanation of low apparent affinity of mitochondria to ADP in these cells by its rapid consumption due to high oxidative activity and by intracellular diffusion problems. However, short treatment of fibers with trypsin decreased this constant value to 40–70 μ M, confirming the earlier proposition that mitochondrial sensitivity to ADP in vivo is controlled by some cytoplasmic protein. Phantom cardiomyocytes which contain mostly mitochondria and cytoskeleton and retain the normal shape, showed also high apparent K_m values for ADP. Therefore, they are probably the most suitable system for studies of cellular factors which control mitochondrial function in the cells in vivo. In these phantom cells anti-desmin antibodies did not change the kinetics of respiration regulation by ADP. However, in skinned fibers from the heart and m. soleus of transgenic desmin-deficient mice some changes in kinetics of respiration regulation by ADP were observed: in these fibers two populations of mitochondria were observed, one with usually high apparent K_m for ADP and the second

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one with very low apparent K_m for ADP. Morphological observations by electron microscopy confirmed the existence of two distinct cellular populations in the muscle cells of desmin-deficient mice. The results conform to the conclusion that the reason for observed high apparent K_m for ADP in regulation of oxidative phosphorylation in heart and slow twitch skeletal muscle cells in vivo is low permeability of mitochondrial outer membrane porins but not diffusion problems of ADP into and inside the cells. Most probably, in these cells there is a protein associated with cytoskeleton, which controls the permeability of the outer mitochondrial porin pores (VDAC) for ADP. Desmin itself does not display this type of control of mitochondrial porin pores, but its absence results in appearance of cells with disorganised structure and of altered mitochondrial population probably lacking this unknown VDAC controlling protein. Thus, there may be functional connection between mitochondria, cellular structural organisation and cytoskeleton in the cells in vivo due to the existence of still unidentified protein factor(s). © 1997 Elsevier Science B.V.

Keywords: Mitochondrion; Respiration; Heart; Skeletal muscle; Regulation; Adenosine diphosphate; Diffusion; Cytoskeleton

1. Introduction

Ultrastructural studies have revealed multiple connections between cytoskeleton elements and mitochondria in different types of cells [1–13]. These connections were clearly demonstrated by Penman group by using embedment-free electron microscopy [11,12]. Numerous earlier electron microscopic studies have revealed close connection between intermediate filaments and mitochondria in various types of the cells [1,3,5,7,8,13]. For cardiac cells, several groups of authors have shown that very often desmin filaments may be seen in close connection to the mitochondria in the heart cells [7,8]. On the other hand, studies of the mechanisms of regulation of mitochondrial respiration in the cells in vivo have shown that in cardiac and in slow-twitch skeletal muscle the value of apparent K_m for ADP is very high but can be decreased significantly by disruption of the outer mitochondrial membrane or by selective digestion of some cytoplasmic protein systems by trypsin or other proteases [14–17]. Also, the affinity of mitochondria to ADP can be increased by an order of magnitude by mitochondrial isolation [17]. These results led to an assumption that the permeability of the porin pores (VDAC) in the mitochondrial outer membrane for ADP (and probably for ATP) in these cells in vivo may be controlled (modulated) by some cytoplasmic proteins, hypothetically related to the cytoskeleton [16,17]. Clearly, for understanding the cellular mechanism of regulation of mitochondrial respiration and energy fluxes, it is of major importance to identify this protein structure which may control the permeability of the porin pores in the mitochondrial outer membrane, and also the possible role of ADP diffusion inside the cells. Equally impor-

tant is to understand the functional significance of the connections of cytoskeleton elements to mitochondria which have often been observed by electron microscopy. The results of detailed study of some of these problems are reported here. They allow to conclude that there is definite relationship between structural organisation of the cell and mitochondrial functional properties in vivo, but desmin itself does not seem to be a factor directly controlling VDAC.

2. Materials and methods

2.1. Construction of the targeting vector, generation and identification of desmin knock-out mice

To disrupt the desmin gene, one targeting vector was constructed in which an *Escherichia coli* LacZ gene was inserted inframe into the first exon of the desmin gene. A neomycin-resistance gene and an HSV thymidine kinase gene were added to allow positive and negative selections. Details of the construction of the desmin targeting factor and production of targeting ES cell lines have been described previously [18]. The modified ES cells in which one desmin allele had been targeted by homologous recombination were microinjected into 3.5-day-old C57BL/6j blastocysts. Two independent ES cell clones were able to colonize the germ line and transmitted the mutated allele to their progeny. Heterozygous mice showed no obvious anatomical or behavioral defects. Desmin knock-out mice were produced by intercrossing heterozygous mice. Homozygous mice were less strong and fatigued more easily. Identification of homozygous (Des $-/-$), heterozy-

gous (Des + / -) and control mice was carried out by southern blot analysis as described previously [18]. Absence of desmin mRNA and protein in the homozygous (Des - / -) mice were verified by northern blot and western blot analysis as described previously [18].

2.2. Isolation and culturing of adult cardiac myocytes

Male Wistar rats weighing 300–350 g were used in all experiments. Calcium-tolerant myocytes were isolated by perfusion with a collagenase-containing medium in a Langendorff apparatus under sterile conditions. The hearts were perfused by about 100 ml of a calcium free solution (initial solution) at a perfusate flow rate 12–14 ml/min, pH 7.3, 37°C, saturated with a gas mixture 95% O₂–5% CO₂ and containing, in mM: NaCl 70 mM, KCl 10 mM, KH₂PO₄ 4 mM, NaHCO₃ 15 mM, MgCl₂ 1 mM, glucose 11 mM, sucrose 120 mM and pyruvate 2 mM. Then the hearts were perfused during about 30 min at 4 ml/min with the same solution to which 1% bovine serum albumin (BSA) and 1 g/l of collagenase D (Boehringer) were added. After that ventricles were placed on Petri dishes (100 mm in diameter) with 10 ml of this solution and 10 ml of initial solution with 2% of BSA. The cardiomyocytes were then gently dissociated by rubber-polisman and pipette suction and reversing action. The suspension of cardiomyocytes was then centrifuged at 30 × *g* for 60 s and myocytes were washed three times and sedimented for 10 min in initial solution with 2% of BSA. Cardiomyocytes were then progressively transferred into calcium-tolerant medium by three washes and sedimentation with mixing of initial solution with 2% of BSA and Sigma medium 199 in ratios 3/1, 3/2 and 3/3, respectively. About 1 to 1.5 × 10⁶ calcium tolerant cardiomyocyte in 15 ml of culture medium 0 (Sigma 199 added of insulin 10⁻⁹ M, sucrose 55 mM, Hepes 20 mM, NaHCO₃ 10 mM, Penicillin 100 IU/ml and streptomycin 0.1 mg/ml) were placed into one Petri dish and incubated at 37°C in atmosphere containing 5% CO₂ and 95% air. After 2–3 h of culture the cardiomyocytes were washed once with culture medium 0. The rod shape calcium-tolerant cardiomyocytes were then incubated for 18 h until saponin treatment.

2.3. Production of phantom cardiomyocytes

To prepare phantom cardiomyocytes without myosin [19], the cardiomyocytes were sedimented from incubation medium and suspended in solution A (see below). For permeabilization of the sarcolemma, saponin was added in concentration of 50 µg/ml and the suspension was incubated for 30 min with slight stirring, and washed by sedimentation 5 times in solution B (control permeabilized cardiomyocytes) or in solution C when phantom cardiomyocytes were prepared. Washed cardiomyocytes were suspended in solution D, containing 800 mM KCl to extract myosin, and incubated for 30 min. After that, phantom cardiomyocytes were sedimented and washed again 5 times in solution B. The extraction of myosin was controlled by determination of the protein concentration in suspension before extraction and in extracts after sedimentation. Usually, the extracts contained 15–20% of the total protein.

In experiments with cardiomyocytes, centrifugation was never used to avoid the damage of the cells.

Antidesmin antiserum was produced in rabbits using purified rat desmin and characterised by the Outherlony test, direct ELISA and immunofluorescence labelling of cardiac cells as described before [20].

2.4. Preparation of skinned muscle fibers

Skinned fibers were prepared according to the method described earlier [14–17]. The animals (Wistar line rats or transgenic mice) were anaesthetised with sodium pentobarbital (50 mg/kg body weight, i.p.) and treated with heparin (1500 IU/kg body weight, i.v.), chest opened and hearts when still beating excised and put into cooled solution A. Cooled hearts were cut into halves and muscle strips (2–4 mm long and 1–1.5 mm in diameter, 5–7 mg of wet weight) cut from endocardium of left ventricles along fiber orientation to avoid mechanical damage of the cells. Muscle fiber bundles (3–4 mm long, about 1 mm in diameter) were taken also from m. soleus (oxidative, slow twitch) and m. gastrocnemius white (glycolytic, fast twitch). By using sharp-ended forceps or needles, the muscle fibers were separated from each other leaving only small areas of contact.

After that the fibers were transferred into vessels with cooled (in ice) solution A containing 50 μg of saponin per ml and incubated at mild stirring for 30 min for complete solubilization of the sarcolemma. Permeabilized (skinned) fibers were then washed in solution B for 10 min; this procedure of washing was repeated two more times to remove completely all metabolites, especially trace amounts of ADP. Complete removal of ADP can be easily seen from respiration recordings which should show very reproducible initial State 2 rates (designated as v_o) not sensitive to inhibition by atractyloside (see below).

2.5. Immunofluorescence confocal microscopy of permeabilized and phantom cardiomyocytes

Double labelling of desmin and mitochondria was performed on intact cardiomyocytes and phantoms in suspension. Phantom cardiomyocytes incubated with or without anti-desmin antibodies were treated for immunofluorescence studies after the experiments on determination of kinetics of respiration determination. We used a modified method of Granger and Lazarides [21]. The fixation of the cells was achieved by incubating the cells during 15 min in freshly prepared 3% paraformaldehyde in phosphate buffered saline (PBS) at 37°C. Permeabilization of intact cardiomyocytes was achieved by incubation in 1% Triton in PBS for 30 min. Phantom cardiomyocytes did not need to be treated by Triton since they were already permeabilized. Then intact and phantom cardiomyocytes taken from respiration experiments in which anti-desmin antibodies had not been added were incubated with polyclonal antidesmin antibodies for 30 min at 37°C. Then all samples including phantom cardiomyocytes which had been incubated with anti-desmin antibodies in experiments, were incubated with rodhamin-conjugated F(ab')₂ secondary antibodies and then with nonylorange solution (0.1 $\mu\text{g}/\text{ml}$) during 15 min. At this time, the cells were washed three times for 15 min in PBS and then in water. Finally the labelled cells in suspension were deposited on glass coverslips and mounted in a mixture of mowiol and glycerol to which 1,4-diazobicyclo-(2,2,2)-octane was added. Samples were observed by confocal microscopy performed with a LSSM410 Zeiss confocal microimaging system equipped with a plan apo x 63 oil immersion, NA 1.40 objective lens. The micro-

graphs shown in this work represent optical sections 0.5 μm thick.

2.5.1. Transmission electron microscopy

For electron microscopy muscles were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 for 2 h. Following post-fixation for 1 h at 4°C in 2% osmium tetroxide and alcohol dehydration, the samples were embedded in polybed 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate on a LKB 2168 ultrastainer and observed in a Jeol 200EX-1 electron microscope.

2.6. Determination of the kinetics of respiration regulation by ADP in skinned fibers and cardiomyocytes

The rates of oxygen uptake were recorded by using the two-channel high resolution respirometer (Oroboros Oxygraph, Paar KG, Graz, Austria) or Yellow Spring Clark oxygen electrode in solution B, containing respiratory substrates (see below) and 2 mg/ml of BSA. Determinations were carried out at 25°C, solubility of oxygen was taken as 215 nmol/ml [14].

2.6.1. Solutions

Composition of the solutions used for preparation of skinned fibers and for oxygraphy was based on the information of the ionic contents in the muscle cells cytoplasm [22].

Solution A contained, in mM: CaK₂EGTA 2.77, K₂EGTA 7.23, MgCl₂ 6.56, dithiothreitol (DTT) 0.5, potassium 2-(N-morpholino)ethansulfonate (K-MES) 50, imidazole 20, taurine 20, Na₂ATP 5.3, phosphocreatine 15, pH 7.1 adjusted at 25°C.

Solution B contained, in mM: CaK₂EGTA 2.77, K₂EGTA 7.23, MgCl₂ 1.38, dithiothreitol (DTT) 0.5, K-MES 100, imidazole 20, taurine 20, K₂HPO₄ 3 and pyruvate 5 (or glutamate 5) + malate, 2, pH 7.1 adjusted at 25°C.

Solution C contained, in mM: taurine 20, dithiothreitol 0.5, MgCl₂ 10, ATP 10, K-MES 80, Hepes 50, pH 7.1 adjusted with KOH.

Solution D contained, in mM: taurine 20, dithiothreitol 0.5, MgCl₂ 10, ATP 10, KCl 800, Hepes 50, pH 7.1 adjusted with KOH.

Solution KCl contained in mM: KCl 125, Hepes 20, glutamate 4, malate 2, Mg-acetate 3, KH₂PO₄ 5,

EGTA 0.4 and DTT 0.3, pH 7.1 adjusted at 25°C and 2 mg of BSA per ml was added.

2.6.2. Reagents

All reagents were purchased from Sigma (USA) except ATP and ADP which were obtained from Boehringer (Germany).

2.6.3. Analysis of the experimental results

The results were analysed by using Enzwitter program for enzyme kinetics. The values of kinetic parameters are expressed as mean values with standard deviation for at least 5 experiments.

3. Results

3.1. Analysis of the regulation of respiration by ADP in different types of muscle cells in vivo

Short time (30 min) treatment of the isolated cardiomyocytes or muscle fibers with a detergent saponin in low concentration of 40–70 $\mu\text{g/ml}$ allows very selectively to dissolve the surface membrane of the cell and remove soluble cytoplasmic proteins, due to high affinity of saponin to cholesterol which content is high in this membrane [10,11,23–25]. Under these conditions, mitochondria preserve their normal morphology, properties and localisation in the cells [10,23–25]. Recent studies of kinetics of regulation of mitochondrial respiration in these permeabilized cells showed that in contrast to the isolated mitochondria, which show very high affinity to ADP, mitochondrial affinity for ADP in vivo is decreased in cardiac and slow twitch but not in fast twitch skeletal muscle [26,27]. This very interesting phenomenon was studied further in this work. Fig. 1 illustrates the tissue specificity of the regulation of the respiration rate of skinned fibers from different muscles of the rat by external ADP. The apparent K_m values for ADP in regulation of respiration calculated from these dependences are very remarkably different for skinned fibers from the heart and m. soleus on the one hand, and gastrocnemius on the other hand. Similar dependences were assessed for many other types of the muscles of the rat, and Fig. 2A summarizes the results of all this work, showing a quantitative relationship between the apparent K_m for ADP

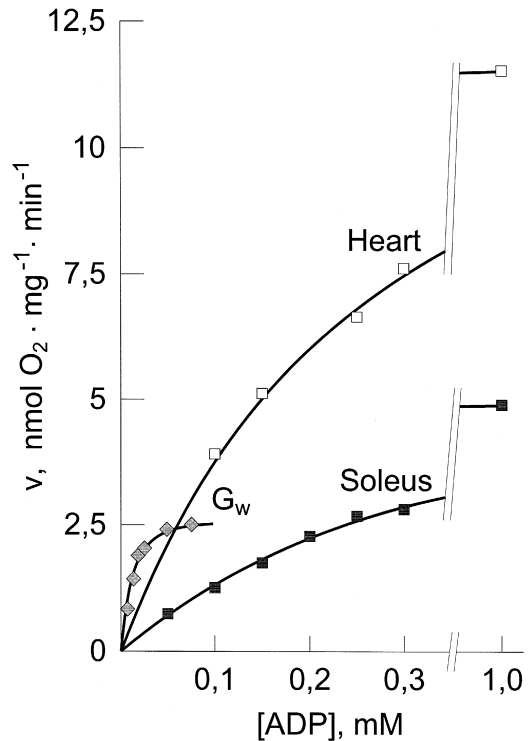


Fig. 1. The respiration rates of skinned fibers from rat heart, soleus and gastrocnemius white muscles as functions of ADP concentration in the medium.

in skinned fibers and the V_{\max} , the maximal respiration rate. Very clearly, in the fibers with high oxidative activity – V_{\max} exceeding 5 nmol of oxygen consumed per min per mg of dry weight – the apparent K_m for ADP is very high, around 300 μM , and in all fibers studied with low oxidative activity the apparent K_m for ADP in vivo is not different from the value of this parameter for isolated mitochondria in vitro, both being between 10–20 μM .

Looking at this relationship, one may think that probably the very high apparent K_m values for ADP in the fibers from muscles with high oxidative activity might be the result of rapid consumption of ADP which diffusion into the cells may be somehow limited by, let us say, incomplete solubilization of sarcolemma, or that it may be the result of mitochondrial clustering. If this explanation is true and the high values of the apparent K_m for ADP are simply artefacts, they should decrease to in vitro values or those in fast skeletal muscle if the respiration in fibers with high oxidative activity is partially inhib-

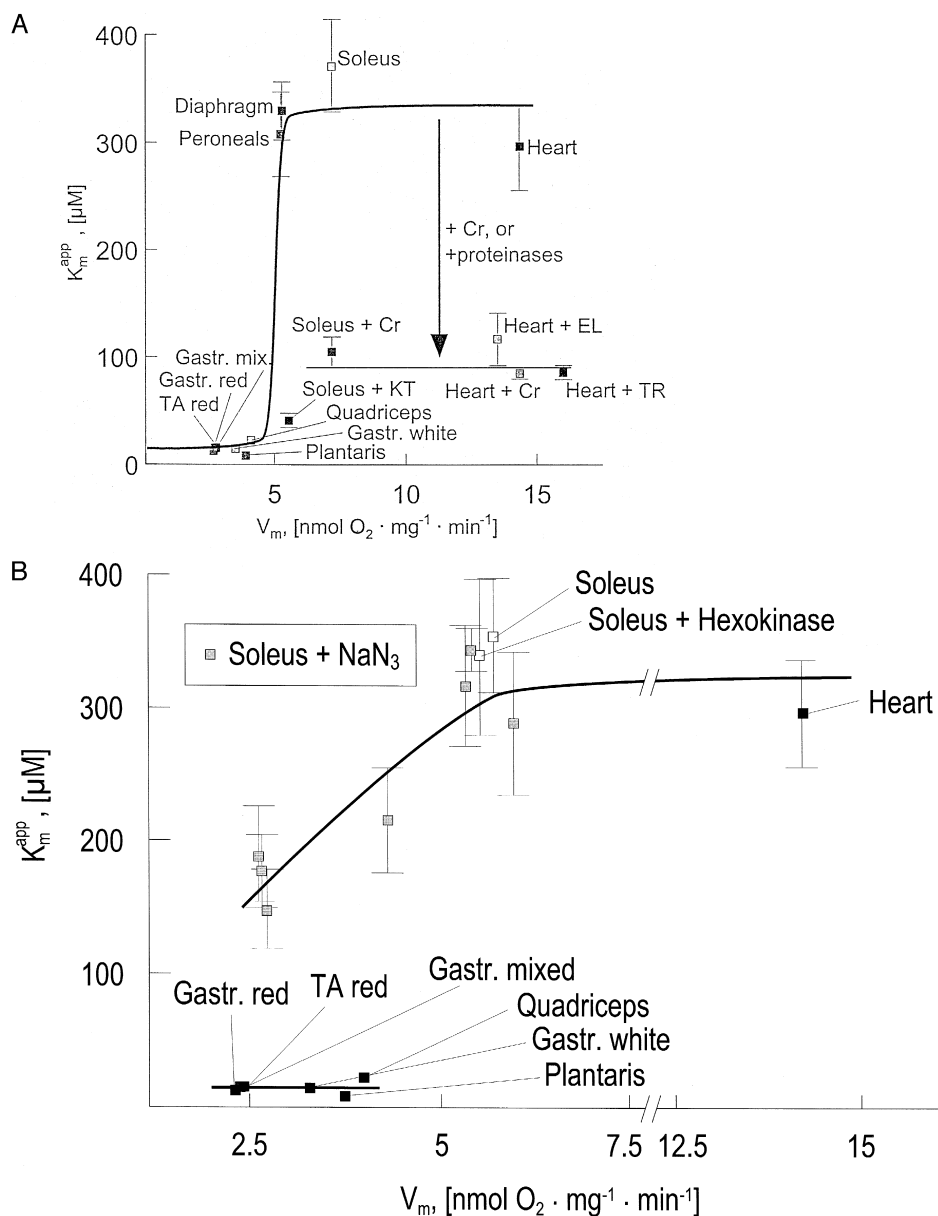


Fig. 2. (A) Summary of the studies of the respiration regulation by ADP in skinned fibers from different muscles of the rat. The phenomenological relationship between apparent K_m for ADP and maximal respiration rate under different conditions are shown. The affinity of mitochondria in the cells in highly oxidative muscles such as heart and slow twitch soleus skeletal muscle, diaphragm and peroneals is very low (apparent K_m very high) but is increased (apparent K_m decreased) in the presence of creatine (20 mM) due to activation of coupled mitochondrial creatine kinase reactions [31] or after the treatment of fibers with proteolytic enzymes trypsin (TR), chymotrypsin (KT) or elastase (EL) for 15 min at the concentration of proteases 0.125 mg/ml. On the contrary, in fast twitch skeletal muscle such as gastrocnemius white, red or mixed, tibialis anterior (TA) red, quadriceps and plantaris the affinity to ADP is initially very high – apparent K_m for ADP is not different from that for isolated mitochondria in vitro. (B) The effect of inhibition of the respiration of the skinned fibers from rat soleus muscle on the apparent K_m for ADP. The respiration in soleus skinned fibers was decreased by adding NaN_3 to the concentration up to 0.3 mM. In some experiments without inhibition the glucose, 10 mM, and high activity of hexokinase, 5 IU, were added to show that even if the rate of ADP regeneration is increased by several orders of magnitude, the apparent K_m value is not decreased. For comparison, the apparent K_m values for fibers from rat heart and fast twitch skeletal muscles plantaris, quadriceps, tibialis red and gastrocnemius red, white and mixed are shown.

ited. Fig. 2B shows that this does not happen: using increasing concentrations, from zero up to 0.3 mM, of sodium azide, we decreased the maximal rate of mitochondrial respiration in m. soleus to the values even lower than those in fast skeletal muscle, but this did not decrease too much the value of apparent K_m for ADP which stayed around 200 μM for fibers from m. soleus. Some decrease of apparent K_m values for ADP, from 300 to 200 μM , may have several interpretations (see Section 4). Taking into account that the apparent K_m for ADP is the same in fibers from heart and m. soleus in spite of almost three-time difference in V_{max} , we may exclude the explanation for high apparent K_m for ADP in regulation of mitochondrial respiration in aerobic muscles, such as heart and soleus, and also in liver and brain [28] by rapid consumption of ADP and its diffusion problems.

Another possible intuitive explanation for high value of apparent K_m for ADP could be that in fast skeletal muscle with low oxidative activity the rates of the actomyosin ATPase reaction and thus the rate of ADP delivery to mitochondria are much higher than in slow twitch muscle this creating the difference in the apparent K_m for external ADP (this again includes an assumption of some diffusion problems for ADP). To check this possibility, we increased the rate of ADP regeneration in skinned fibers from soleus by adding hexokinase, 5 IU per 3 ml and 10 mM glucose. The value of the apparent K_m for ADP in respiration regulation was not changed (see Fig. 2B). Which means that in these fibers with relatively

slow actomyosin ATPase, the ATPase activity is already higher than the ATP synthase activity. This is in concord with our earlier data which showed that even after extraction of myosin (“ghost” fibers) the MgATPase activity was sufficient to regenerate ADP since the apparent K_m for ADP was not changed by the added hexokinase + glucose system [15]. Also, treatment of the fibers with trypsin is known to decrease the actomyosin ATPase activity [29], but

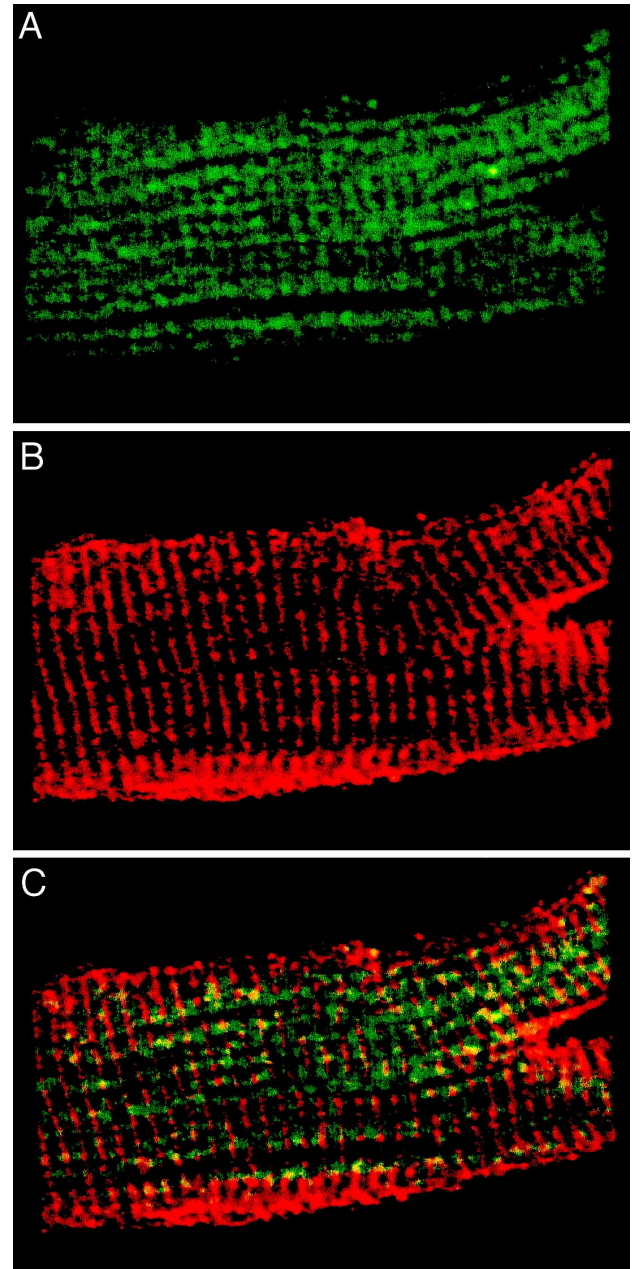


Fig. 3. Double labelling immunofluorescence confocal microscopy of phantom cardiomyocytes. (A) The labelling of mitochondria by nonylorange (see Section 2). The green colour is that of nonylorange associated with mitochondrial membranes. Parallel rows of mitochondria are seen. Note also the interesting striation pattern of mitochondrial position (1 cm equals 6 μm). (B) The labelling of desmin in phantom cardiomyocytes. Phantom cells were incubated in solution B with antidesmin antiserum (dilution 200 times) for 1 h before respiration experiments, then one part of cells was taken for respiration experiments and another part of cells was fixed and incubated with rodhamine-conjugated F(ab')₂ secondary antibodies. Desmin (red colour) is mostly associated with the Z-lines (1 cm equals 6 μm). (C) The superposition of (A) and (B). We see that mitochondria (green colour) are always between the Z-lines whereby desmin labelling (red colour) is clearly seen (1 cm equals 6 μm).

under these conditions the affinity of mitochondria to ADP is in fact increased – apparent K_m for this substrate is decreased several times (Fig. 2A).

Thus, the observed phenomenon – decreased affinity of mitochondrial respiration to ADP in vivo – is not an artefact due to rapid consumption of ADP in the cells with high oxidative activity. On the contrary, it is a tissue specific phenomenon, which can be observed only if mitochondrial respiration is studied in vivo by using the permeabilized cell and skinned fiber technique and which is not observed when mitochondria are isolated from the cells.

Most probably, it reflects some important intracellular mechanisms of regulation of mitochondrial function intrinsic for the cells in vivo. Since the value of apparent K_m for ADP is decreased several times by treatment of fibers by trypsin (see Fig. 2A), the phenomenon must be related to expression of some specific cytoplasmic, extramitochondrial proteins which control the permeability of the porin pores in mitochondrial outer membrane (VDAC) [16,17,30]. Indeed, when ADP is produced in coupled mitochondrial creatine kinase reaction in intermembrane space in the presence of creatine, the apparent K_m for ADP is again decreased due to the increased turnover of adenine nucleotides in the coupled reactions (Fig. 2A, see [15–17,31]). Also, disruption of the mitochondrial outer membrane by well controlled hypoosmotic treatment strongly increases the affinity of mitochondria in vivo to ADP up to the values seen in vitro [30,31].

Some important peculiarities of this type of respiration regulation were revealed in studies of ghost cardiomyocytes.

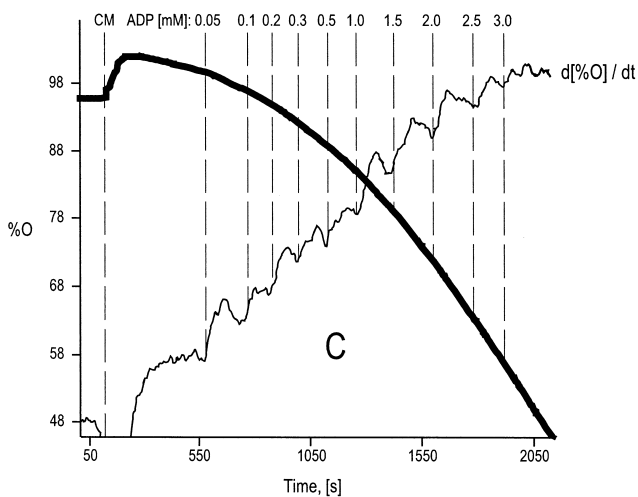
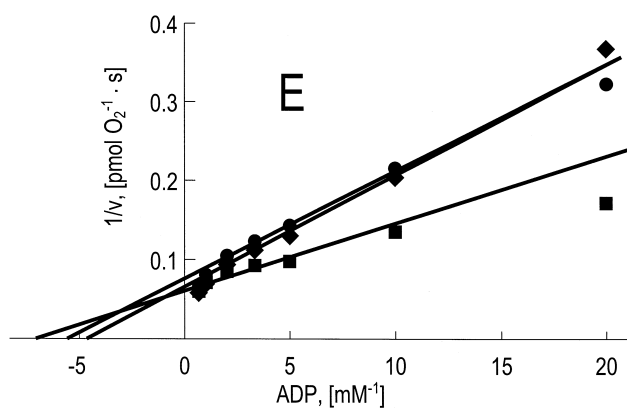
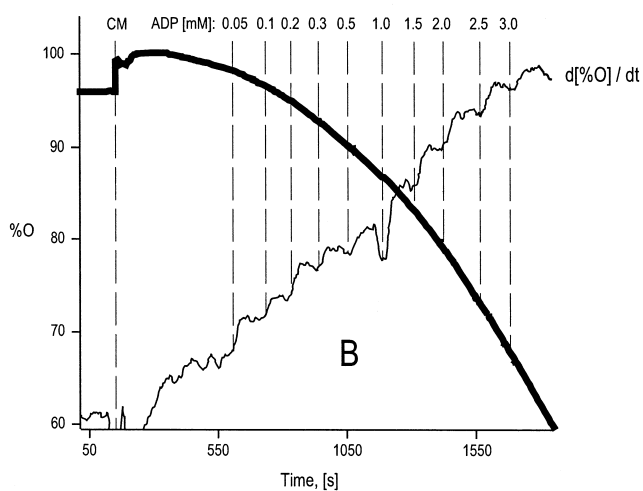
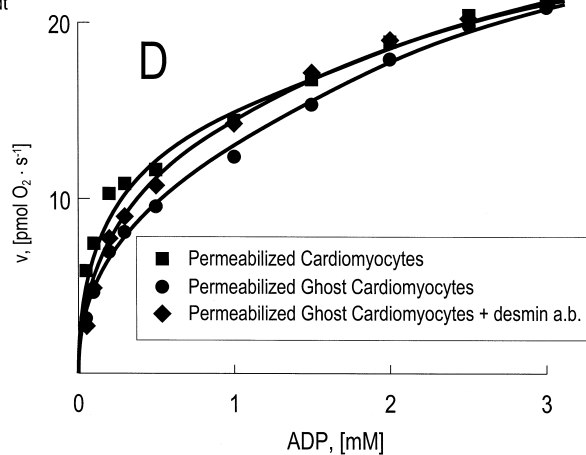
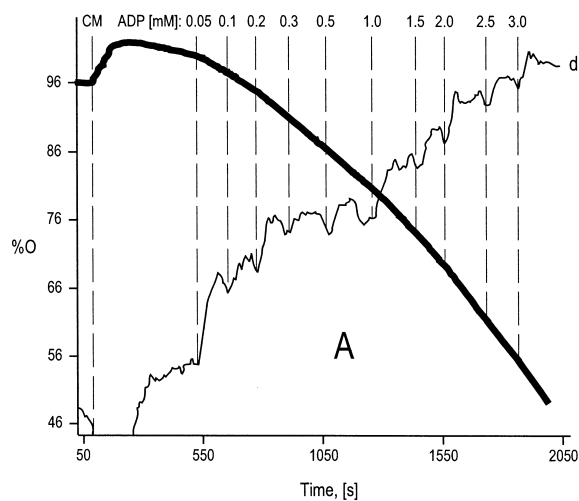
3.2. Analysis of the respiration regulation in “ghost” (phantom) cardiac cells

Fig. 3 shows the confocal microscopic images of isolated cardiomyocytes after permeabilization of the sarcolemma by saponin and after extraction of myosin from these permeabilized cells. The resulting cells are

called “ghost” or “phantom” cells [19]. The sarcolemma of these cells is completely permeabilized since antidesmin antibodies used before or during respiration experiments, without any additional permeabilization, perfectly stain the cells mostly on the Z-lines (see striations in Fig. 3B). In spite of removal of myosin, the “phantom” cardiomyocytes retain the normal cell shape due to preservation of cytoskeletal system, and abundant mitochondria (green colour) in normal position forming parallel longitudinal rows (Fig. 3A) as normally seen in electron microscopy [8]. One more interesting feature of mitochondrial localization in the phantom cells can be seen in Fig. 3A: it appears that mitochondrial positions if looked by confocal microscopy of permeabilized cells follow the pattern of striation of myofibrils. In Fig. 3C the confocal microscopic images after double immunolabelling for desmin (red colour) and mitochondria are superimposed showing perfectly regular structures in “phantom” cardiomyocytes. This is a system consisting of cytoskeleton, including actin filaments, mitochondria and also other intracellular membrane structures such as sarcoplasmic reticulum not extractable by KCl solution. This double labelling for mitochondria and desmin gives an impression that mitochondria are positioned in the space between structures which are stained by antidesmin antibodies and correspond to Z-lines of myofibrils. The results shown in Fig. 3 demonstrate that in fact the cytoskeletal system of cardiomyocytes is a very strong structure: after permeabilisation of sarcolemma and treatment with solution with high ionic strength and complete removal of thick myosin filaments, this structure maintains the normal shape of cardiomyocytes; moreover, it preserves the precise localisation of mitochondria in the cells.

Fig. 4 shows the functional characteristics of mitochondria in these phantom cells. In all cases – permeabilized cells, phantom cardiomyocytes before and after binding of the antidesmin antibodies – the respiratory characteristics of mitochondria are the same (Fig. 4A–D), and the apparent K_m for ADP in

Fig. 4. Respiration rates of permeabilized cardiomyocytes (A), phantom cardiomyocytes (B) and phantom cardiomyocytes after binding of antibodies against desmin (C) as the dependences on the ADP concentration in the medium. Oxygraph traces and their first derivatives (respiration rates) are shown. (E) Linearisation of the dependences given in (D) in double reciprocal plots.



regulation of respiration (Fig. 4E) is high, between 200 and 250 μM . Since big molecules of myosin have been extracted and antibodies against desmin easily penetrate into these single isolated cells, any diffusion problem across the sarcolemma seems to be

excluded. The addition of trypsin increases the respiration rate transitorily (Fig. 5). The decrease of the respiration rate after the initial activation in response to trypsin is most probably due to rapid proteolysis of ATPases and thus the decrease of the rate of ADP

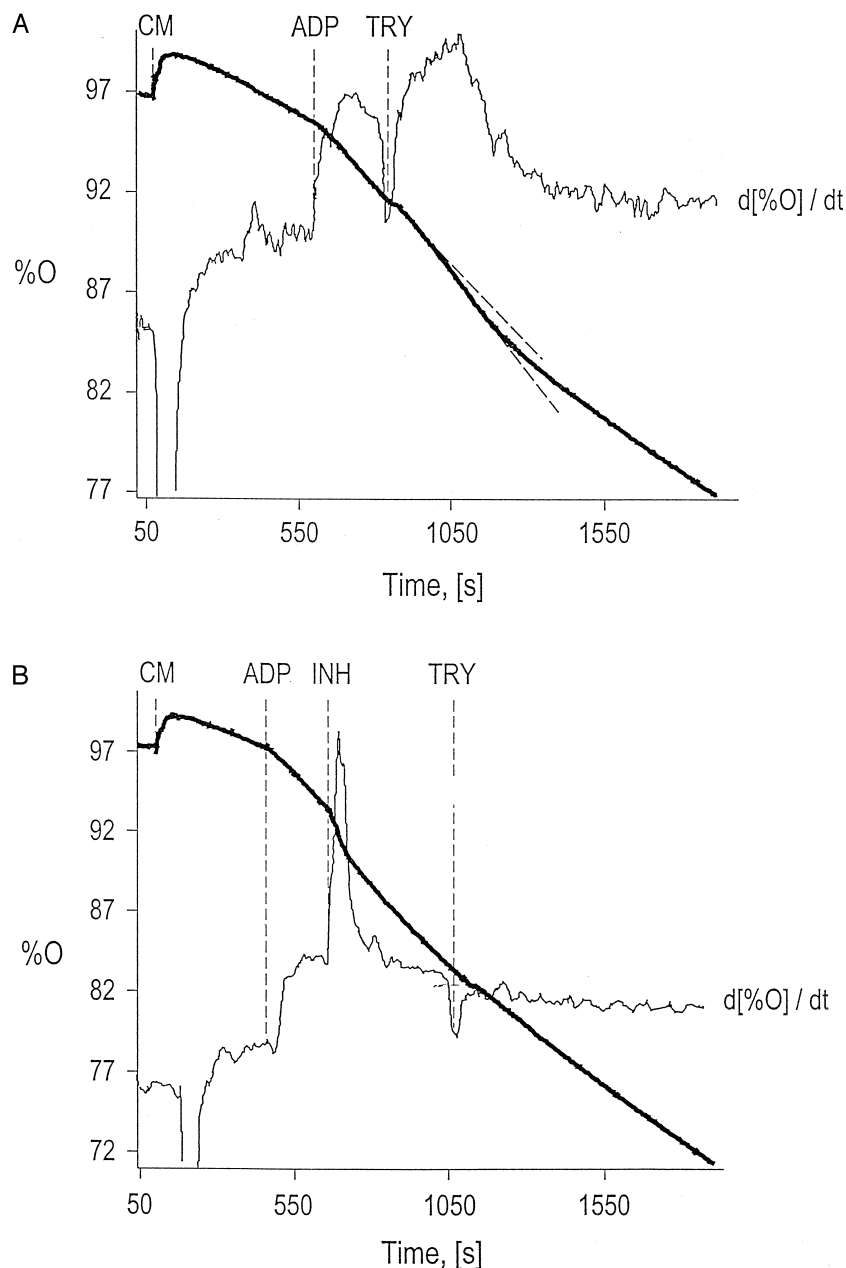


Fig. 5. Increase of respiration rate of isolated permeabilized cardiomyocytes after treatment with trypsin. (A) Oxygraph traces and their first derivatives (respiration rates) of permeabilized cardiomyocytes (CM) after addition of ADP, 0.1 mM, and trypsin (TRY), 12.5 $\mu\text{g}/\text{ml}$. (B) Oxygraph traces and their first derivatives (respiration rates) of isolated permeabilized cardiomyocytes treated with trypsin inhibitor from bovine pancreas, 25 $\mu\text{g}/\text{ml}$ and then by trypsin (TRY), 12.5 $\mu\text{g}/\text{ml}$.

regeneration. Thus, these “phantom” systems contain the protein which is obviously non-soluble and associated with cytoskeletal structures and which seem to control the permeability of mitochondrial porin pores for adenine nucleotides. Since polyclonal antibodies against desmin added into the medium do not block the ADP-stimulated respiration, most probably desmin itself is not directly connected to these porin channels.

However, to study in more detail the role of desmin in this phenomenon, and the possible importance of the intermediate filament-mitochondrial connections for regulation of energy fluxes in the cell, we further studied the mechanism of regulation of respiration in muscle cells of desmin-deficient transgenic mice.

3.3. Alterations of the kinetics of regulation of respiration by ADP in the heart and skeletal muscle cells *in vivo* of transgenic desmin-deficient mice

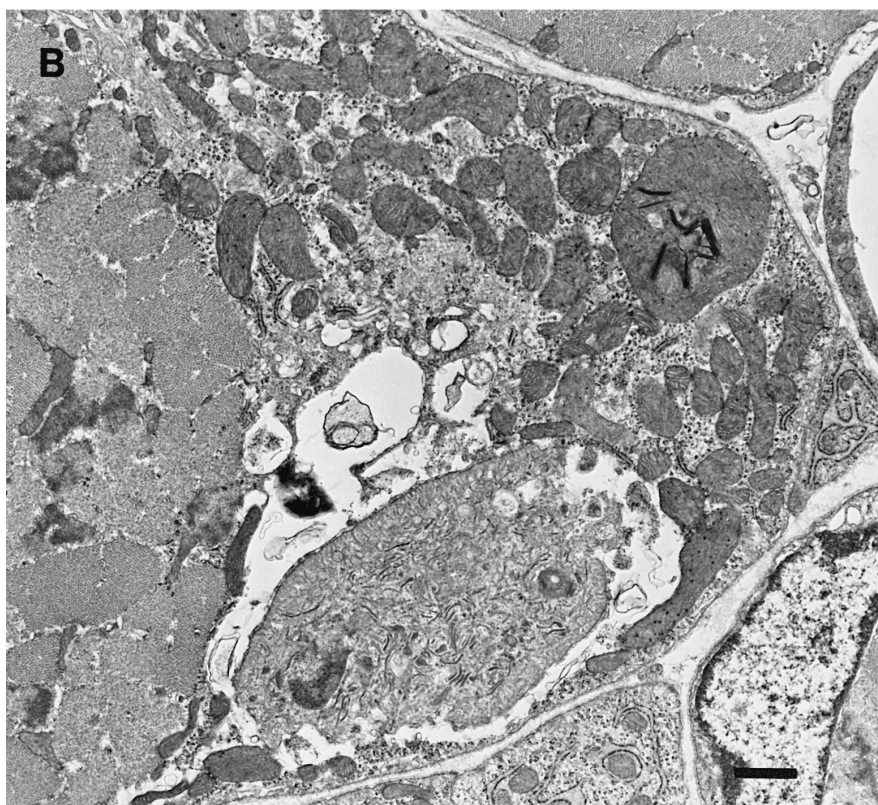
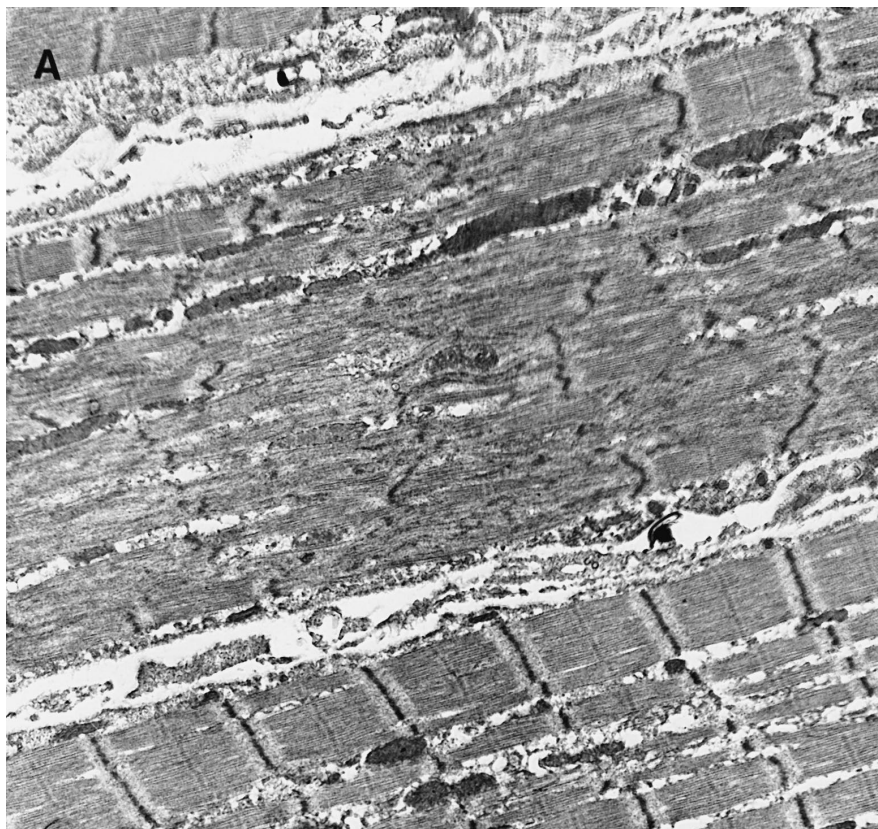
In order to investigate the possible role of desmin filaments in control of mitochondria, gene targeting was used to produce mice bearing a null mutation in the desmin gene [18]. Earlier, we have shown that skeletal, cardiac and smooth muscle form in the Des $-/-$ mice, but morphological abnormalities were observed as demonstrated by disorganised, distended and nonaligned fibers [18]. Here we examined more closely, using electron microscopy, the ultrastructure of heart and soleus muscles from newborn to 12 week old mutant mice. Lack of desmin results in focal degeneration of muscle fibers followed by re-

Table 1

Kinetic parameters for respiration regulation in skinned fibers from muscles of transgenic mice deficient in desmin

Type of muscle, parameter of respiration	Type of mouse		
	Wild	Heterozygote, Des $+/-$	Homozygote, Des $-/-$
<i>I. Heart</i>			
K_m (ADP), μM			
Control	167 ± 38	220 ± 44	I: 178 ± 15 II: 33 ± 6
+ Creatine	68 ± 31	70 ± 18	83 ± 10
V_{\max} , nmole $\text{O}_2/\text{mg} \cdot \text{min}$			
Control	17.5 ± 3	17 ± 5	I: 22 ± 2.1
+ Creatine	16 ± 3	13 ± 3	18 ± 7
<i>II. M. Soleus</i>			
K_m (ADP), μM			
Control	242 ± 65	262 ± 50	I: 226 ± 70 II: 14 ± 7
+ Creatine	—	—	—
V_{\max} , nmole $\text{O}_2/\text{mg} \cdot \text{min}$			
Control	8.1 ± 3.9	9 ± 5	I: 4.2 ± 2.2
+ Creatine	—	—	—
<i>III. M. Gastrocnemius</i>			
K_m (ADP), μM			
Control	4.9 ± 2.9	6.4 ± 1.7	11 ± 6
+ Creatine	—	—	—
V_{\max} , nmole $\text{O}_2/\text{mg} \cdot \text{min}$			
Control	2.8 ± 1.5	—	2.5 ± 0.9
+ Creatine	—	—	—

The mean values and standard deviations are shown for 7–11 experiments. V_{\max} is rate of respiration dependent on ADP (v_o subtracted).



Heart oxygraph recordings :

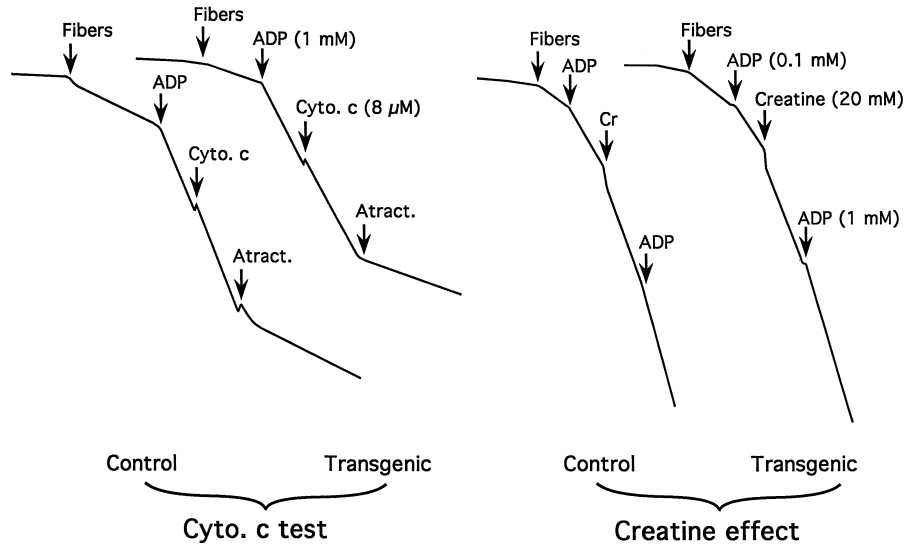


Fig. 7. Respiration recordings of the skinned fibers from the wild (control) and desmin deficient (transgenic) mice hearts. Cytochrome c test was used to investigate the state of the mitochondrial outer membrane in the skinned fibers (see the text). Creatine test to check the coupling of mitochondrial creatine kinase to adenine nucleotide translocase (see the text). Both controls are identical in wild and transgenic mice.

generation and fibrosis in highly solicited skeletal muscles. Already at one week in the Des $-/-$ mice we observed abnormal sarcomeres with no clear demarcation of I and A bands as well as disintegrated myofibers with sparse filaments in soleus muscle (Fig. 6). Individual muscle fibers were frequently misaligned and disoriented to such an extent that longitudinal and transverse views could be observed side by side in the same section. Z-disc streaming was also observed, that resulted in degeneration of muscle fibers. These included variability in fiber diameter, the presence of central nuclei, fibrosis, macrophage infiltration, necrosis, and the presence of crescent shape sarcolemmal masses. Mitochondria were rounded and had distorted inner membranes.

In cross sections of the Des $-/-$ mice heart the majority of myocytes were seen to contain myofibrils and mitochondria in an ordered pattern similar to that in the Des $+/+$ mice. However, dilatation of the sarcotubular system was often apparent, the intercalated discs were altered, the myofibrils were often stretched and fragmented. The number of desmosomes was reduced. Mitochondria were often rounded and contained matrix granules not present in normal mitochondria. In conclusion, the appearance of two populations of the muscle cells with normal and abnormal ultrastructural organisation were observed in Des $-/-$ mice. Fig. 6B shows that in the soleus skeletal muscle of these Des $-/-$ mice two populations of mitochondria – those positioned regularly in

Fig. 6. Ultrastructure of soleus muscle from one week old Des $-/-$ mice. Longitudinal (A) and transversal (B) ultrathin sections. In (A), two myocytes are seen. The lower myocyte has a normal regular structure of sarcomere, but the myocyte seen in the upper part of this figure is abnormal: note appearance of irregular sarcomeres, myofibril lesions, Z-discs streaming, no clear demarcation of I and A bands as well as disintegrated fibers with sparse filaments are seen. Moreover, individual muscle fibers were commonly misaligned and disoriented to such an extent that longitudinal and transverse views could be observed side by side in the same section. In (B), note formation of subsarcolemmal population of mitochondria (upper right corner) disconnected from myofibrils and often enlarged. This may represent a population of mitochondria with different affinity for ADP (see the text). Bar = 1 μ m.

Examples of kinetics :

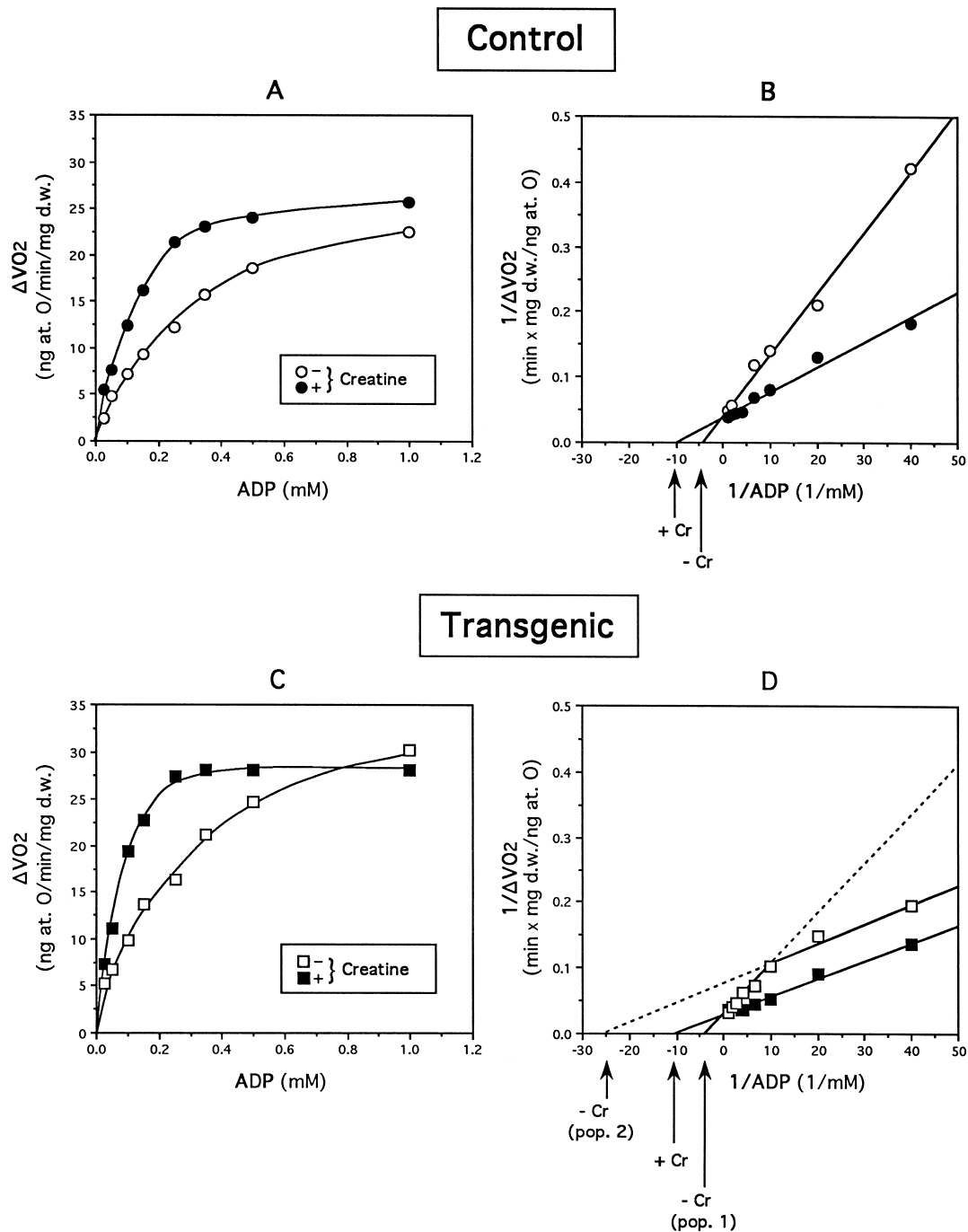


Fig. 8. Alteration of kinetics of respiration regulation in the skinned fibers from the desmin deficient hearts. (A) Respiration rates of cardiac skinned fibers from the wild mouse in the absence (○) and presence (●) of creatine, 20 mM. (B) Linearization of the dependences from (A) in the double reciprocal plots. (C) Respiration rates of the skinned cardiac fibers from Des $-/-$ mice in the absence (□) and presence (■) of creatine, 20 mM. (D) Linearization of the dependences from (C) in double reciprocal plots.

the intermyofibrillar space and those clustered in the subsarcolemmal area, without clear connection to the myofibrils, often enlarged and containing matrix granules – may be sometimes observed even within the same myocyte.

Fig. 7 shows the recordings of respiration – consumption of oxygen – by skinned cardiac fibers from control (wild) and transgenic desmin deficient mice, Des – / –. First, the cytochrome c test was used to investigate the state of the outer mitochondrial membrane. In the KCl medium cytochrome c dissociates from the outer surface of the inner mitochondrial membrane and if the outer mitochondrial membrane is damaged, it leaves mitochondrial intermembrane space, that decreasing the rate of respiration [15]. However, addition of the exogenous cytochrome c completely restores respiration under these conditions. The results of the use of this test for investigation of the state of the outer mitochondrial membrane are shown in the Fig. 7A. This figure shows high rates of respiration stimulated by ADP in saturating concentration which are not changed by addition of exogenous cytochrome c both in fibers from wild and desmin deficient mouse hearts. Thus, in both cases the outer mitochondrial membrane is intact. Fig. 7B shows that in both cases creatine stimulates the respiration at non-saturating ADP concentration to approximately similar degree (Table 1) that showing the presence of normal coupled mitochondrial creatine kinase activity in the desmin-deficient hearts.

However, some clear distinctions between fibers from the heart of the wild type and desmin deficient mice can be seen when the kinetics of respiration regulation by ADP is studied (Fig. 8). This figure shows the respiration rates of the skinned fibers in dependence of the ADP concentration in the surrounding medium in the presence and absence of creatine, 20 mM. In both cases creatine elevates the respiration rates at submaximal ADP concentrations (Fig. 8A and C), due to the presence of the coupled creatine kinase activity [31]. In double reciprocal plots a single straight line is seen in the case of fibers from wild (control) mouse heart (Fig. 8B), giving average values of apparent K_m for ADP equal to 167 μM in the absence and 68 μM in the presence of creatine, respectively (see Table 1) at unchanged V_{\max} values.

However, similar kinetic analysis of respiration

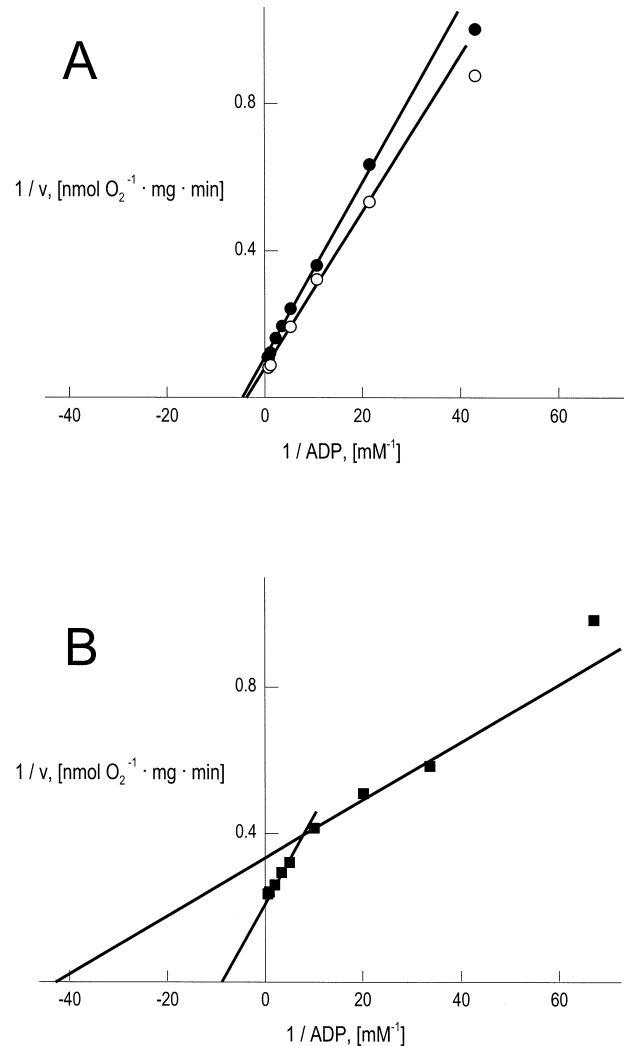


Fig. 9. Alteration of the kinetics of regulation of respiration in skinned fibers from soleus skeletal muscle of the desmin deficient mice. Dependences are given in double reciprocal plots. (A) Respiration of skinned fibers from soleus skeletal muscles of wild (○) and heterozygous (●) mice. (B) Respiration of skinned fibers from m. soleus of homozygous mice.

regulation in skinned cardiac fibers from transgenic Des – / – mice revealed two functionally different populations of mitochondria. This is very clearly seen in Fig. 8D when the data were analysed in double reciprocal plots. In the absence of creatine one population of mitochondria is characterised by high apparent K_m value for ADP similar to that observed in control (wild mice) (Table 1), and the second one characterized by low value of this constant close to that observed for isolated mitochondria in vitro. In

the presence of creatine, due to strong amplifying action of the coupled creatine kinase reaction [31], apparent K_m for the first population is decreased and the difference between the populations is not seen any more (Fig. 8D). Statistically analysed average values of these parameters are given in the Table 1.

Exactly the same phenomenon was seen for the skinned fibers from m. soleus (Fig. 9). In this case, but not for the heart, we observed also significant decrease of the V_{max} value of the population with low affinity to ADP (Table 1).

In fast twitch gastrocnemius muscles we did not find any kinetic differences in the mitochondrial population of the wild and Des $-/-$ mice (Table 1).

4. Discussion

The results of this study show that the high values of apparent K_m for ADP in regulation of the mitochondrial respiration in cardiac and slow twitch muscle cells in vivo are not related to rapid consumption of ADP or its slow diffusion from outside into the cells, but most probably are related to the expression of the unknown protein factor which controls the permeability of porin pores in the mitochondrial outer membrane for adenine nucleotides. Further, the results show that desmin itself does not control the mitochondrial outer membrane permeability for ADP but its complete absence in transgenic Des $-/-$ mice muscle cells induces the appearance of morphologically and functionally distinct mitochondrial population due to disorganized cellular structure.

The results of this work as well as earlier studies of permeabilized cardiomyocytes and skinned fibers show [14–17,26–28,30,31] that in contrast to isolated mitochondria which have, independently from the tissue, very low apparent K_m for ADP, mitochondria in the cells in vivo expose a tissue specificity of the mechanism of regulation of respiration by ADP. Very clearly, this phenomenon is illustrated in Fig. 2 of this work. The tissue specificity of the mitochondrial affinity for ADP excludes the explanation of low affinities to ADP in the cells with high oxidative activity – in the cells of m. soleus, heart, but also liver and brain [28,30] – by limited diffusion of ADP from outside of the cells, since in fast skeletal muscle cells these diffusion problems might be even bigger

than in the cardiac cells due to larger diameter of the cells. In the skinned fibers from heart and m. soleus there is no difference between apparent K_m value for ADP in spite of 3-fold difference in V_{max} , and decrease of the respiration rate from 6.5 to 2.5 nmol/min/mg dry weight due to inhibition of respiration in skinned fibers from m. soleus decreases the apparent K_m value only by 30%, and the lowest value of apparent K_m for ADP still exceeds that for fast skeletal muscle (or mitochondria in vitro) by order of magnitude. This 30% decrease may accommodate some influence of ADP diffusion from outside, but may also be explained by kinetic factors such as redistribution of the control strength between outer and inner mitochondrial membrane systems due to the use of the inhibitors.

The phenomenon of tissue specificity of respiration regulation by ADP also invalidates an explanation of high values of the apparent K_m for ADP in cardiac cells in vivo by the effect of detergent recently proposed by Wiseman et al. [32]. This explanation was based on very early data by Bygrave and Lehninger on submitochondrial particles produced by digitonin [33], but it ignores the differences in detergent concentration used: to produce inner mitochondrial membrane-matrix preparation, digitonin has to be used in the concentration of 6 mg/ml [33] and even those preparations show very high affinity to ADP without any inhibitory effect [34]. For preparation of submitochondrial particles one has to increase the detergent concentration further. However, to produce permeabilized cells or skinned fibers, saponin is classically used in very low concentration to dissolve selectively the sarcolemma. Starting with the studies of Endo and Kitazawa in 1978 and then in very multiple studies, saponin is used for this purpose in concentration of 50 μ g/ml (0.05 mg/ml), that is in concentration 100 times lower than that used to produce inner membrane-matrix preparation from mitochondria [10,11,23–25]. This is due to high content of cholesterol in the plasmalemma of the cells significantly exceeding that in mitochondrial membranes, and due to high affinity of saponin to cholesterol [24]. Many electron microscopic and functional studies including those by Altschuld [25] and Penman groups [10,11] and our earlier studies [15] have shown perfect preservation of intracellular structures, including outer mitochondrial membrane and connections be-

tween cytoskeleton and mitochondria in these permeabilized cells.

The experimental observations that the disrupter of outer mitochondrial membrane by swelling of mitochondria in hypoosmotic conditions [15,30] and treatment of permeabilized cells by proteolytic enzymes both decrease the apparent K_m for ADP are the basis for a conclusion that in cells with high values of apparent K_m for ADP the permeability of mitochondrial outer membrane porin pores for adenine nucleotides is controlled by some cytoplasmic protein or protein structures [16,17]. Since high values of the apparent K_m for ADP are also observed in “ghost”, or “phantom” cardiomyocytes after removal of all soluble proteins and even myosin, one may think that the protein controlling mitochondrial outer membrane permeability for ADP is somehow associated with cellular structures, most probably with cytoskeleton. The “phantom” cardiomyocytes may be taken to be the simplest cellular system deprived of all soluble proteins and myosin in which mitochondrial properties are still similar to those in the cells *in vivo*.

Earlier, Liu and Colombini [35] and Holden and Colombini [36] have found the conserved modulator protein of porin channels (VDAC) in the isolated mitochondrial preparations from different sources. It is not excluded that this conserved protein may have some relation to our cytoskeleton-related protein factor, for example, representing a residual fraction of it contaminating isolated mitochondrial preparations. Moreover, it has been found that type-1 porin (VDAC) is expressed in different cellular compartments, and complexing of VDAC with different modulator proteins may lead to its specific roles at different cell sites [37].

In multiple ultrastructural studies of the cells by using electron microscopy, the connections between mitochondria and cytoskeleton elements have been observed since 1967 [1–13]. Most advanced studies of this problem belong to the Leterrier and Linden groups who have identified microtubule-associated proteins (MAP2) interacting with porin-containing domain of the outer mitochondrial membrane in brain cells [1,9]. In addition, unidentified proteins which were recognized by antibodies to all intermediate filaments subunits were found to be associated with either the mitochondrial surface or with microtubules [1]. These connections have been thought to serve for

dislocation of mitochondria in the cells [1,9,13]. In details, mitochondrial motility and shape changes in several types of living non-muscle cells due to their interactions with cytoskeleton have been reviewed by Bereiter-Hahn and Voth [13]. These authors also described a very interesting phenomenon of delayed morphological response of mitochondria to ATP or ADP micro-injected into living cells, indicating that diffusion of these molecules is about 10 times slower than that of other small hydrophilic molecules [13]. This observation is in concord with our results showing decreased and controlled permeability of adenine nucleotides across mitochondrial membranes resulting in more than 10 times decreased affinity of mitochondria *in vivo* to ADP ([14–17], this work). Penman group has developed a special simple technique for visualization of the connections of cytoplasmic membranes with cytoskeleton [10,11]. For this they used embedment – free electron microscopy which yields high contrast images with no need for staining [10]. To deplete the cells of soluble proteins which confound images of cytoskeleton they used saponin (0.01% v/v, or 100 $\mu\text{g}/\text{ml}$) [10,11] that is practically the same permeabilization technique used by us. The results of their work show many connections of cytoskeleton to mitochondrial membranes [10,11]. These connections are taken to be important for localization of mitochondria in the cells and structural organisation of the cellular systems [11]. Our results confirm this conclusion and show also that these contacts may be important for control of some important mitochondrial function such as affinity to cytoplasmic ADP [14–17].

Since the immunolabelling technique has often revealed the connections between intermediate filaments and mitochondria [3–5,7,8] and in particular between desmin filaments and mitochondria, the desmin intermediate filaments are among the possible candidates for controlling mitochondrial outer membrane permeability for adenine nucleotides *in vivo*.

The results of this study give a very clear answer to the question whether connections of desmin intermediate filaments to mitochondria control their function and whether the desmin is the component of cytoskeletal system that controls the permeability of the outer mitochondrial membrane porin pores for adenine nucleotides. The answer is “no” for the following reasons. (1) Fast twitch skeletal muscle

contain desmin but the permeability of the mitochondrial outer membrane for ADP is not controlled and very high. (2) In the transgenic homozygous Des^{-/-} mice the mitochondrial affinity to ADP in major part of skinned fibers from heart and soleus muscles is still low. (3) In permeabilized rat heart cardiomyocytes binding of anti-desmin antibodies does not change the value of apparent K_m for ADP. We have recently observed that modification of the microtubular system by colchicine or taxol and of microfilaments by cytochalasin also does not change the high values of the apparent K_m in skinned cardiac fibers [28]. These three filaments – microtubules, microfilaments and intermediate filaments are, however, not more than 20% of the cytostructure, according to recent biochemical measurements [11].

However, we have also made another interesting observation. In the heart and m. soleus the high values of the apparent K_m for ADP are seen in the cells which preserve highly organised cellular ultrastructure. This “superstructure” – we may call it so because of its fine organisation (see Fig. 3), and strong resistance to violent treatment – is seen in “phantom” cardiomyocytes in which one observes also high values of apparent K_m for ADP. Mitochondrial position in “phantom” cells follows striated pattern (Fig. 3B) due to spreading of desmin filaments from Z-lines into the space between mitochondria in intermyofibrillar space (Fig. 3C). This is in concord with earlier immunochemical observation by Lockard and Bloom [7] and in fact, in very many electron micrographs, mitochondria (if not clustered in the cells) seem to be separated from each other at the level of Z-line [8]. However, it is not known which proteins, besides desmin, may participate in this kind of structural organization. Morphological observations reported in Fig. 6 of this work show the appearance of cells with morphological abnormalities and disorganized structure in the heart of transgenic mice, and most probably, these structural changes are responsible for appearance of the population of mitochondria with increased affinity to ADP. This shows that probably there is a close connection between structural organisation of the cell and the cellular mechanism of regulation of ADP and energy fluxes. Probably, such a structural disorganization results in dissociation or removal of the VDAC – controlling

factor we are looking for, from mitochondrial surface.

Decreased V_{max} in fibers from soleus muscle may be related to decreased content of mitochondria in these altered cells, or even to their release into medium from subsarcolemmal clusters (see Fig. 6B) during preparation of skinned fibers. In general, this conforms to the previous conclusion that desmin is essential to strengthen the integrity of the cells in skeletal, cardiac and smooth muscle cells [18].

There are two possible explanations for the physiological role of the controlled permeability of the outer mitochondrial membrane for adenine nucleotides *in vivo*. First, this increases the compartmentation of the adenine nucleotides in the cells and the importance of the creatine kinase system in the intracellular energy transfer [31,38]. Second, the cellular structures which control the mitochondrial permeability for ADP may participate in building up the pathway of vectorial ligand conduction for feedback signal transduction in the cells with high energy requirements, as it was proposed in general terms by P. Mitchel [39]. In this case it is only the exogenous ADP for which the permeability of the outer membrane of mitochondria is high in the cells, while there may be a structurally organised pathway for ADP channelling in cytoplasm with participation of creatine kinase and myokinase reactions [16,17,28,30,31]. Very interesting observation has recently been made by Velasco et al. who found that in permeabilized hepatocytes carnitine palmitoyltransferase I in the mitochondrial outer membrane is controlled by extramitochondrial cell components, most probably associated with cytoskeleton, and influenced by cytoskeletal integrity [40].

However, identification of cellular factors which control the permeability of the outer mitochondrial membrane porin pores for adenine nucleotides in the muscle cells with high oxidative activity requires further work and intensive investigations.

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